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REGULATION OF PROKARYOTIC ADENYLYL CYCLASES BY CARBON DIOXIDE

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Running title: Adenylyl Cyclase and Carbon Dioxide.

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SYNOPSIS.

The Slr1991 adenylyl cyclase of the model prokaryote *Synechocystis* PCC 6803 was stimulated two-fold at 20 mM total inorganic carbon at pH 7.5 through an increase in k_{cat} . A dose response demonstrated an E.C.₅₀ of 52.7 mM total inorganic carbon at pH 6.5. Slr1991 was activated by CO₂ but not HCO₃⁻. CO₂ regulation of adenylyl cyclase was conserved in the CyaB1 adenylyl cyclase of *Anabaena* PCC 7120. These adenylyl cyclases represent the only identified signalling enzymes directly activated by CO₂. The findings prompt an urgent reassessment of the activating carbon species for proposed HCO₃⁻ activated adenylyl cyclases.

Keywords: adenylyl cyclase, cAMP, carbon dioxide, bicarbonate, *Synechocystis*

INTRODUCTION.

Inorganic carbon (Ci) is fundamental to the physiology of all organisms. Carbon dioxide and bicarbonate ions exist in a pH dependent equilibrium and are the major biologically active forms of Ci. CO₂ and HCO₃⁻ are vital to such diverse physiological processes as photosynthetic carbon fixation [1], pH homeostasis [2], and carbon metabolism [3]. Study of Ci biology is essential to understand these vital physiological processes. Relatively little is known of the signalling mechanisms through which prokaryotic and eukaryotic cells directly detect CO₂/HCO₃⁻ fluctuations [4]. The identification of Ci activated signalling molecules and their role in physiology is fundamental to understanding the diverse roles of Ci in biology. Currently, no signalling enzymes directly activated by CO₂ are known.

The mammalian soluble adenylyl cyclase (sAC) synthesizes the second messenger adenosine 3', 5'-cyclic monophosphate and is stimulated by HCO₃⁻ [5, 6]. It was observed that HCO₃⁻ regulation of adenylyl cyclase (AC) was conserved in a cyanobacterial AC, CyaC of *Spirulina (Arthrospira) platensis*, which had significant sequence homology in the AC domain to sAC [6]. More recently, an active site Asp→Thr polymorphism in the Class III AC family has been proposed as a marker for HCO₃⁻ responsiveness [7]. On this basis proposed HCO₃⁻ responsive ACs are predicted to be widespread among the genomes of prokaryotes and eukaryotes [8]. To date, Ci regulation of AC has been confirmed in prokaryotes as diverse as *Anabaena* PCC 7120, *Mycobacterium tuberculosis*, *Stigmatella aurantiaca*, and *Chloroflexus aurantiacus* [7, 9]. An implicit assumption is made in the literature that the activating Ci ligand for AC is HCO₃⁻ on the basis that the ionic form is more likely to bind in the active site than CO₂. Identification of the activating carbon ligand for AC is essential to validate or question the relevance of significant recent literature in the field.

The photosynthetic cyanobacteria are an excellent model for investigating Ci signalling through AC as hypothesized HCO₃⁻ responsive ACs are widespread in these organisms and Ci has clearly defined roles in their physiology. Here we demonstrate that the single Class III AC, Slr1991 (Cya1), of the unicellular cyanobacterium *Synechocystis* PCC 6803 is activated by Ci. Furthermore, we demonstrate, surprisingly, that the activating ligand for this enzyme is CO₂ and not HCO₃⁻. A previously characterized proposed HCO₃⁻ regulated AC, CyaB1 of *Anabaena* PCC 7120, is also proven to respond to CO₂ rather than HCO₃⁻. This work provides the first evidence for AC as a CO₂ activated signalling molecule. This original finding prompts an immediate reassessment of the true activating carbon species in reported HCO₃⁻ responsive ACs.

MATERIALS AND METHODS.

Recombinant proteins – DNA corresponding to amino acids 120-337 of *slr1991* was isolated by PCR from genomic DNA of *Synechocystis* PCC 6803, subcloned into pQE30, and fitted with an N-terminal MRGSH₆GS affinity tag. Constructs were confirmed by double-stranded sequencing. Slr1991₁₂₀₋₃₃₇ protein was expressed in *Escherichia coli* M15 [pREP4] cells at 25 °C, for 3 h with 300 µM isopropyl β-D-1-thiogalacto-pyranoside. Pelleted cells were washed (50 mM Tris-HCl 8.5, 1 mM EDTA), resuspended (50 mM Tris-HCl pH 8.5, 250 mM NaCl, 10 mM 1-thioglycerol), lysed by sonication (1 x 150 s), and protein purified from the supernatant with Ni²⁺-nitrilotriacetic acid (NTA, Qiagen) as previously described [10]. CyaB1₅₉₅₋₈₅₉ protein was generated as previously described [10]. Primer sequences are available on request.

Adenylyl cyclase assays - AC assays were performed at 40 °C in a final volume of 100 µl and typically contained 50 mM buffer, 2 mM MnCl₂, 2 mM [2,8-³H]-cAMP (150 Bq), and [α -³²P]ATP (25 kBq) as substrate, if not stated otherwise [11]. Protein concentrations were adjusted to maintain substrate utilization at less than 10%. Kinetic constants were determined over a concentration range of substrate of 1-100 µM (Mn²⁺-ATP). The following buffers were used at pH 6.5 (2-[N-morpholino]ethanesulfonic acid; MES), pH 7.0-7.5 (3-[N-morpholino]propanesulfonic acid), pH 8.0-8.5 (Tris-[hydroxymethyl]aminomethane hydrochloride, and pH 9.0 (2-[N-cyclohexylamino]ethanesulfonic acid). Enzyme, buffer, and substrate were all prepared at the appropriate pH for the required assay. CO₂ was quantitated by titration against NaOH. Assay pH was stable over a period of at least 40 minutes. All errors correspond to the standard error of the mean. If absent, errors are smaller than the symbol used to depict the data point.

RESULTS AND DISCUSSION.

The *cyaI* (slr1991; <http://www.kazusa.or.jp/cyano/Synechocystis>) gene of the unicellular cyanobacterium *Synechocystis* PCC 6803 encodes an enzyme consisting of a single FHA (Forkhead Associated) domain and a Class III AC domain that contains an Asp→Thr polymorphism associated with a putative HCO₃⁻ responsiveness [7, 12]. We expressed the AC domain of Slr1991 as a purified recombinant protein (Figure 1a). The purified wild type protein had a significant AC specific activity in the presence of both Mg²⁺-ATP (154 ± 2.0 pmol cAMP mg⁻¹ min⁻¹, n=8) and Mn²⁺-ATP (5816 ± 87 pmol cAMP mg⁻¹ min⁻¹, n=8) under optimal conditions (pH 9.5, 40 °C, 0.3 mM ATP, 8 μM protein).

The Slr1991₁₂₀₋₃₃₇ protein had a pH optimum of 9.5 and a temperature optimum of 40 °C. The enthalpy of activation (E_A) derived from the linear arm of an Arrhenius plot using Mn²⁺-ATP was 33.5 ± 1.4 kJ mol⁻¹ (n=6). We investigated whether Slr1991₁₂₀₋₃₃₇ was regulated by Ci with a view to determining the identity of the activating species, CO₂ or HCO₃⁻. Slr1991₁₂₀₋₃₃₇ specific activity was stimulated two-fold by 20 mM total Ci (1.2 mM CO₂/18.8 mM HCO₃⁻) at pH 7.5 compared to Cl⁻. Stimulation was independent of cation and robust to 95% confidence intervals (Figure 1b). A previous report had not observed stimulation of Slr1991 by Ci at pH 7.5 [13]. We noted that an extended assay period (40 mins) was required to observe robust Ci activation of Slr1991 at pH 7.5. Although Masuda and Ono do not report assay time, this is the most likely cause of the discrepancy.

We determined the kinetics of activation of Slr1991₁₂₀₋₃₃₇ by Ci (Table I). Slr1991₁₂₀₋₃₃₇ showed Michaelis-Menten kinetics in the presence of both Cl⁻ and Ci. The K_M value for Mn²⁺-ATP was greater in the presence of Ci than Cl⁻ but V_{max} values were proportionately greater for Ci than Cl⁻. The overall result was that Ci increased turnover rate (k_{cat}). A dose response curve with increasing Ci was performed at a reduced pH 6.5 to eliminate problems with enzyme inhibition at >20 mM total Ci at pH 7.5 in the presence of Mn²⁺-ATP (Figure 2). The experiment revealed a maximum eight-fold stimulation with an apparent E.C.₅₀ for Ci of 52.7 ± 1.0 mM (n=6) (20.4 mM CO₂/32.3 mM HCO₃⁻).

We investigated the response of Slr1991₁₂₀₋₃₃₇ to total Ci at varying pH to gain insight into whether the enzyme is responsive to CO₂ and/or HCO₃⁻. The experiment was performed using Mg²⁺-ATP as substrate as Mg²⁺ co-factor is more soluble than Mn²⁺ in the presence of Ci at alkaline pH. Intriguingly, relative stimulation (Ci:NaCl) varied from 1.1 at pH 8.5 (0.3 mM CO₂/39.1 mM HCO₃⁻/0.6 mM CO₃²⁻) to 2.4 at pH 6.5 (15.5 mM CO₂/24.5 mM HCO₃⁻) (Figure 3a). This is consistent with a role for CO₂ as opposed to HCO₃⁻ as the activating carbon species but may also be due to altered protonation status of the enzyme limiting the ability of Slr1991 to respond to HCO₃⁻ at elevated pH. We therefore sought direct evidence

for regulation of Slr1991₁₂₀₋₃₃₇ by CO₂ and/or HCO₃⁻ by analysis under conditions of Ci disequilibrium when a single predominant carbon species, CO₂ or HCO₃⁻, is present at a defined pH. We exploited the fact that acquisition of the equilibrium between CO₂ and HCO₃⁻ is significantly lowered at reduced temperature in the absence of carbonic anhydrase and is a well established method for identifying the Ci substrate for CO₂/HCO₃⁻ fixing enzymes [14]. We followed the acquisition of the CO₂/HCO₃⁻ equilibrium by measuring the pH of a weakly buffered (5 mM) MES solution on addition of 20 mM CO₂ or 20 mM NaHCO₃ in the presence or absence of carbonic anhydrase at 0°C (Figure 3b). On the basis of this data we defined conditions for assaying AC under conditions of disequilibrium using 20 mM CO₂ or HCO₃⁻ as a 10 second assay period at 0°C after addition of Ci. Under these conditions, Ci is predominantly in the form added to the assay (CO₂ or HCO₃⁻) and has not significantly advanced toward the equilibrium determined by assay pH (clamped with 100 mM MES). Control experiments demonstrated that under the conditions used for the assay final pH was equivalent when either CO₂, HCO₃⁻, or Cl⁻ were added demonstrating that any observed stimulation was due to addition of Ci and not a change in assay pH (data not shown).

We assayed Slr1991₁₂₀₋₃₃₇ under conditions of Ci disequilibrium and observed, surprisingly, that CO₂ but not HCO₃⁻ stimulated the enzyme (Figure 3c). We investigated whether this highly significant result was unique to Slr1991 or of more general significance. The CyaB1₅₉₅₋₈₅₉ protein of *Anabaena* PCC 7120 was previously shown to respond to HCO₃⁻/CO₂ but the activating species not proven [7]. Consistent with the findings for Slr1991₁₂₀₋₃₃₇, CyaB1₅₉₅₋₈₅₉ was also stimulated by CO₂ but not HCO₃⁻ under conditions of Ci disequilibrium (Figure 3d).

This data demonstrates that for at least two randomly selected prokaryotic ACs, Slr1991 and CyaB1, the activating carbon species is dissolved CO₂ and not the more ably binding HCO₃⁻ species. These enzymes therefore represent the first identified signalling molecules demonstrated to respond directly to CO₂. HCO₃⁻ regulation of AC has been proposed but not proven for enzymes from species as diverse as *Spirulina platensis*, *Cryptococcus neoformans*, *Candida albicans*, *Chloroflexus aurantiacus*, and mammals [6, 9, 15, 16]. An urgent examination of these systems is required to prove whether the ACs defined from these species respond to HCO₃⁻ or to CO₂ as described here.

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FIGURE LEGENDS.

Figure 1. Adenylyl cyclase activity of purified recombinant Slr1991₁₂₀₋₃₃₇. **A**, Purification of recombinant Slr1991₁₂₀₋₃₃₇ (SDS-PAGE analysis, Coomassie Blue staining). 1.5 µg of protein was applied and molecular mass standards (in kDa) are indicated. **B**, Slr1991₁₂₀₋₃₃₇ specific activity (n=8) in presence of 20 mM total Ci/salt (0.6 µM protein, 20 µM Mn²⁺-ATP, pH 7.5).

Figure 2. Response of wild Slr1991₁₂₀₋₃₃₇ to Ci. Slr1991₁₂₀₋₃₃₇ specific activity (n=6) at increasing total Ci (1.5 µM protein, 20 µM Mn²⁺-ATP, pH 6.5, Na⁺ as cation, total salt made up to 200 mM with NaCl).

Figure 3. Activation of adenylyl cyclase by CO₂. **A**, The ratio of the specific activities of Slr1991₁₂₀₋₃₃₇ when assayed in the presence of 40 mM total Ci or NaCl at varying pH (8 µM protein, 1 mM Mg²⁺-ATP, 20 mM Mg²⁺). The inset shows the percentage of total Ci made up by CO₂ and HCO₃⁻ over the pH range tested. **B**, Change in pH of a 5 mM MES solution (starting pH 6.4) on addition of 20 mM NaHCO₃ (arrow) in the presence (squares) or absence (triangles) of 132 U carbonic anhydrase at 0°C. **C**, cAMP produced by Slr1991₁₂₀₋₃₃₇ under conditions of Ci disequilibrium (50 µM Slr1991₁₂₀₋₃₃₇ protein, 0°C, 10 secs, 20 mM CO₂/NaHCO₃/NaCl, 100 mM MES pH 6.5, 150 µM Mn²⁺-ATP). **D**, cAMP produced by CyaB1₅₉₅₋₈₅₉ under conditions of Ci disequilibrium (38 µM CyaB1₅₉₅₋₈₅₉ protein, 0°C, 10 secs, 20 mM CO₂/NaHCO₃/NaCl, 100 mM MES pH 6.5, 150 µM Mn²⁺-ATP, n=6).

Table I. Kinetic parameters for Slr1991₁₂₀₋₃₃₇. 0.6 μ M protein was assayed at pH 7.5 in the presence of 20 mM salt (n=6).

Parameter	Addition	
	Cl ⁻	HCO ₃ ⁻
V _{max} (nmol cAMP mg ⁻¹ min ⁻¹)	0.74 ± 0.01	1.13 ± 0.03
K _M [ATP] (μ M)	11.4 ± 0.7	16.2 ± 1.3
Hill Slope	1.01 ± 0.01	1.03 ± 0.03
k _{cat} (min ⁻¹)	0.018	0.027

Figure 1a.

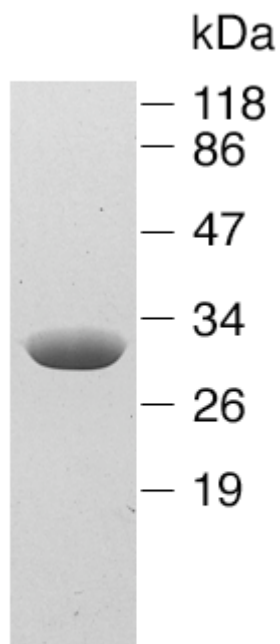


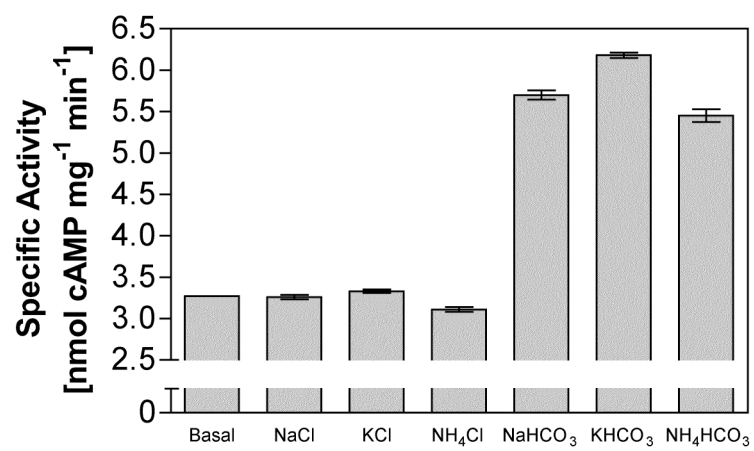
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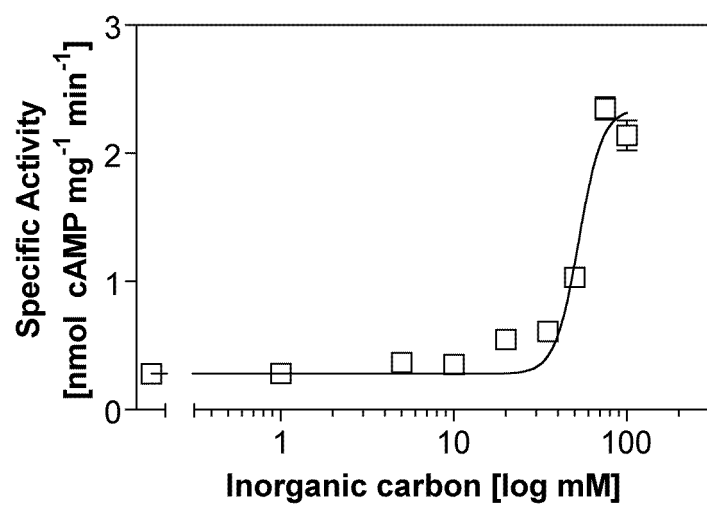
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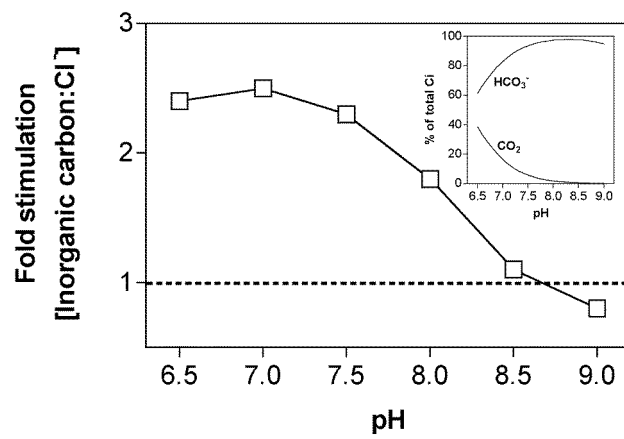
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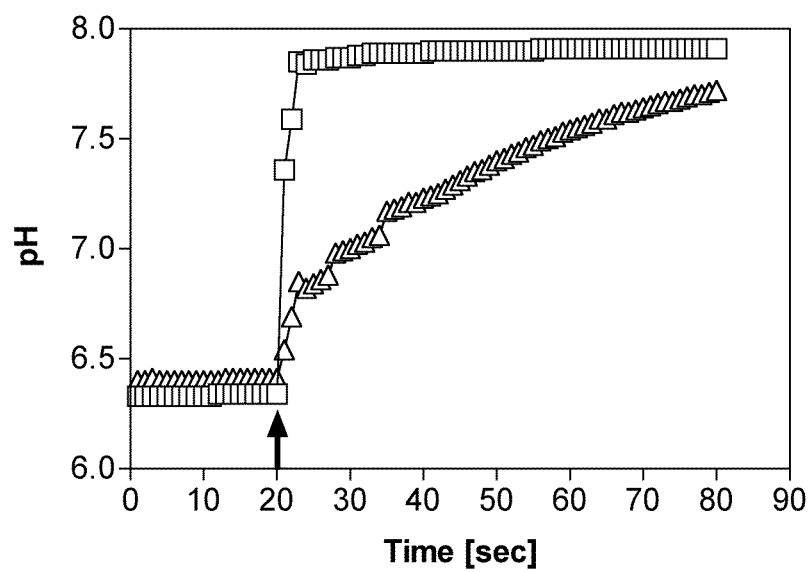
Figure 3b.

Figure 3c

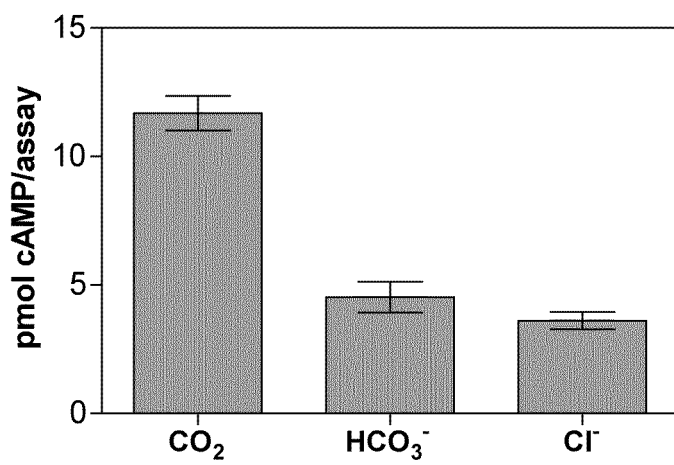


Figure 3d